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Technical note

Effects of dehydration-induced structural and material changes on the apparent modulus of cancellous bone

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ABSTRACT

Dehydration is known to cause an increase in the elastic modulus of bone tissue. However, it also causes structural changes (i.e. shrinkage) which can themselves significantly alter the mechanical properties, particularly in cancellous bone. The current study attempts to estimate the contribution of these two competing factors to the net change of dehydration on the apparent modulus of bovine cancellous bone. Cylindrical cores from the lumbar vertebrae were tested in tension, while hydrated and again after dehydration. The bone volume fractions (BV/TV) were measured in both conditions. The results indicate that the average overall increase in the apparent modulus after dehydration is $14 \pm 14\%$ (mean $\pm$ SD), which represents the net effect of a 27% increase in modulus due to increased tissue modulus offset by a modulus decrease of 13% due to reductions in bone volume fraction. These observations underscore the need to consider both structural and material changes when comparing hydrated and dehydrated mechanical behaviour.

1. Introduction

Bone tissue is composed of three primary constituents: mineral, collagen and water. This composite nature of bone tissue makes it difficult to understand how the phases interact with one another to produce the observed mechanical properties. Therefore, one of these three constituents is often removed in order to study its relative contribution to the overall behaviour.

Water has been found to affect both the mechanical properties and the structure of bone (Table 1). At the macroscopic level, dehydration results in increased apparent modulus, but decreased strength, fracture strain and fracture toughness [26,37]. Nanoindentation studies performed at the microstructural level report increased elastic modulus and hardness in dehydrated tissues [27,16]. The structure of the bone also changes when water is removed: the spacing between collagen fibrils decreases [18] and a macroscopic shrinkage of the bone dimensions occurs [13]. If dehydrated bone is to be studied, then both of these effects must be considered; however, there are no guidelines for assessing the relative contributions of structural and material changes.

Cancellous bone is an ideal candidate for separating out these individual effects because the magnitude of the tissue stiffening and structural changes is much larger than in cortical bone. For example, nanoindentation studies by Akhtar et al. [2] in bovine cancellous bone revealed an increase in the tissue modulus of 28% (from 14.3 to 18.3 GPa) due to drying. In the absence of structural changes, the resulting increase in apparent modulus is expected to be proportional to the increase in the tissue modulus [34]. However, Lievers et al. [20] recently reported a reduction in bone volume fraction (BV/TV) of approximately 16% in bovine cancellous bone dehydrated at room temperature. Given the power-law relationship between apparent modulus and bone volume fraction [14], such a decrease would be accompanied by a drop in the apparent elastic modulus. As illustrated in Fig. 1, the net change in the apparent modulus of cancellous bone will be the summation of these two effects.

The goal of the current study is to estimate the relative contributions of structural and material changes caused by dehydration to the change in the apparent elastic modulus of cancellous bone. Cylindrical cores of bovine cancellous bone from the lumbar vertebrae were tested in hydrated and then dehydrated conditions using an end-constrained uniaxial testing protocol. The bone volume fraction of the samples was measured in both conditions. By comparing the hydrated and dehydrated power-law regressions relating apparent modulus to bone volume fraction, the relative contribution of structural and tissue changes was estimated in can-

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Table 1
Recent papers examining the role of water in calcified tissues.

Paper	Summary
Macroscopic-level studies	
Currey et al. [11]	Dehydration increases modulus and bending strength in deer antler
Yan et al. [37]	Dehydration decreases fracture toughness in bovine cortical bone
Nyman et al. [26]	Dehydration decreases toughness, increases stiffness and strength in human femoral cortical bone
Kruzic et al. [17]	Dehydration decreases fracture toughness in elephant tusk
Yamashita et al. [36]	Dehydration decreases viscoelasticity in human femoral cortical bone
Microstructre-level studies	
Wolfram et al. [35]	Dehydration increases nanoindentation modulus in human cancellous bone
Utku et al. [33]	Dehydration causes anisotropic contraction of equine metatarsal osteonal lamellae
Akhtar et al. [2]	Dehydration increases nanoindentation modulus in bovine cancellous bone
Hoffler et al. [16]	Dehydration increase nanoindentation modulus in human cortical bone

cellous bone. An improved understanding of the interdependency of these two factors will be valuable for studies where the mechanical properties of hydrated and dehydrated bone are compared, and will provide further insight into the different roles of water in calcified tissues.

2. Materials and methods

Seventeen fresh adult bovine lumbar vertebrae were obtained from a local slaughterhouse. Only half vertebrae were available due to the butchering process. One cylindrical core, 8.3 mm in diameter, was removed in the cranial-caudal direction from half vertebrae L4 to L6 using a diamond-coated coring tool (Starlight Industries; Rosemont, PA). The bone cores were then frozen for up to 4 weeks [5]. Prior to further specimen preparation, the cores were thawed and soaked in phosphate-buffered saline (PBS) for 12 h to ensure full rehydration [32]. Following rehydration, the cores were cut to nominal 28 mm lengths using a jeweller's saw and bone marrow was removed from approximately 3 mm of each end using a jet of tap water (InterPlak, Conair). The ends of the specimens were then embedded in epoxy (Technovit 3040, Heraeus Kulzer) using the split mould design of Lievers et al. [22] and promptly returned to the PBS solution. A total of 51 specimens were prepared with an exposed gauge length of 16 mm.

Specimens were tested in elastic tension, on the day of embedding, employing an Instron 5500 R (Model 1122) with a 500 kg load

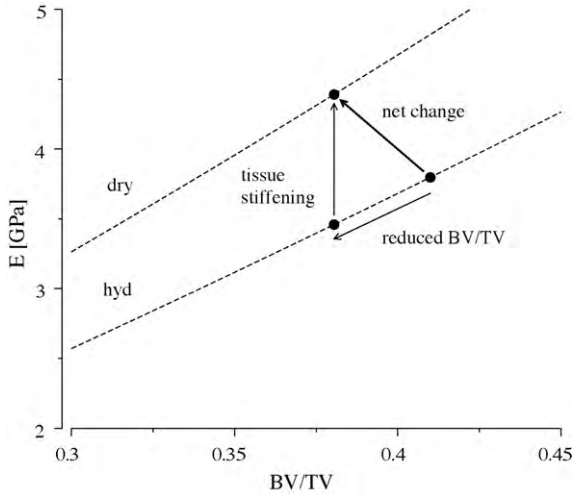

Fig. 1. An idealized plot of apparent elastic modulus (E) as a function of cancellous bone volume fraction (BV/TV). The dotted lines represent the power-law curves relating E and BV/TV for both hydrated (hyd) and dehydrated (dry) conditions. The net change in the macroscopic properties due to dehydration is comprised of an apparent modulus reduction due to volumetric shrinkage and an increase in the tissue modulus.

cell. The tensile and compressive elastic moduli of cancellous bone have been shown to be equivalent [7,25,31], so tension was chosen over compression because of previous work by the authors [22,21]. Specimens were loaded to an engineering strain of 0.15% at a rate of 0.1 mm/min and strain was measured using a gauge-mounted 6 mm extensometer (Epsilon Technology Corp.; Jackson, WY) attached with dental elastics, also referred to as orthodontic rubber bands. Two successive tests were performed, with the extensometer affixed in two opposing positions on the specimen, in order to account for architectural variability and frame-induced bending. Specimens were kept immersed in saline until they were tested, but no hydration or humidity control were employed. Total time from removal to the completion of testing was approximately 8–10 min.

The apparent modulus was calculated based on a linear fit from 0 to 0.1% strain for each test, and the average value of the two tests was reported as the hydrated apparent modulus for each specimen (E^{app}_{hyd}). The appropriateness of the linear fit was evaluated using the R^2 value and specimens with tests having an $R^2 < 0.993$ were removed.

Specimens were left to dry in air at room temperature until a steady-state level of dehydration was reached (typically 3 days), at which point they were placed in a vacuum desiccator for a further 24 h only to ensure a steady-state had been reached. The specimens were then re-tested in tension, as outlined above, in order to measure the dehydrated apparent moduli (E^{app}_{dry}). Testing was not performed under vacuum or under humidity control. The specimens were subsequently refrozen.

To obtain measures of volume and density, the specimens were thawed and nominal 6 mm long sections were extracted from the center of the gauge region using a cut-off saw (Accutom-5, Struers). The bone marrow was removed with a jet of tap water and the specimens were defatted for 24 h in a 1:1 (v/v) chloroform/methanol solution under agitation [9,19]. Water jet cleaning was repeated after defatting to remove any remaining fatty tissue. The specimens were then centrifuged (IEC Micromax RF, Thermo Electron Corporation) at $100 \times g$ for 10 min in PBS to ensure complete hydration of the bone [19]. The submerged mass (m_{sub}) of the defatted specimens was measured while suspended in PBS and the apparent volume (V^{app}_{hyd}) was calculated from the average of five height (h_{hyd}) and diameter (D_{hyd}) measures performed using digital calipers.

The specimens were then blotted dry and centrifuged at $100 \times g$ for 10 min, in a dry centrifuge tube containing absorbent paper [19,32], and weighed to obtain the hydrated mass (m_{hyd}). The specimens were subsequently dried, as described above (i.e. roughly 3 days in air, and 24 h in a vacuum desiccator), to obtain the dehydrated mass (m_{dry}). The dehydrated apparent volume (V^{app}_{dry}) was again calculated from the average of five height (h_{dry}) and diameter (D_{dry}) measurements.

The average change in macroscopic height (Δh) and diameter (ΔD) of the excised cores was evaluated using:

$$\Delta h = \frac{h_{dry} - h_{hyd}}{h_{hyd}}, \qquad \Delta D = \frac{D_{dry} - D_{hyd}}{D_{hyd}}. \tag{1}$$

Paired t-tests were also used to compare the effects of dehydration on the height and diameter. A value of $p < 0.05$ was deemed significant, while $0.05 \geq p < 0.1$ was deemed a trend.

Using Archimedes' method, the hydrated tissue volume (V_{hyd}^{tiss}) was calculated from:

$$V_{hyd}^{tiss} = \frac{m_{hyd} - m_{sub}}{\rho_{PBS}} \tag{2}$$

where $\rho_{PBS} = 1.003\,\mathrm{g/cm^3}$ [3]. Lievers et al. [20] have demonstrated, by comparing helium pycnometer measurements with Archimedes' method, that the volume of cancellous bone dehydrated at room temperature (V_{dry}^{tiss}) can be calculated as follows:

$$V_{dry}^{tiss} = \frac{m_{dry} - m_{sub}}{\rho_{PBS}}. \tag{3}$$

Hydrated and dehydrated cancellous bone volume fractions (BV/TV_{hyd}, BV/TV_{dry}) were calculated using:

$$\frac{BV}{TV_{hyd}} = \frac{V_{hyd}^{tiss}}{V_{hyd}^{app}}, \qquad \frac{BV}{TV_{dry}} = \frac{V_{dry}^{tiss}}{V_{dry}^{app}}. \tag{4}$$

Finally, hydrated and dehydrated apparent densities:

$$\rho_{hyd}^{app} = \frac{m_{hyd}}{V_{hyd}^{app}}, \qquad \rho_{dry}^{app} = \frac{m_{dry}}{V_{dry}^{app}} \tag{5}$$

were also calculated to allow for comparison with other studies. Paired t-tests were performed to assess the statistical changes in the apparent moduli (E^{app}), the bone volume fractions (BV/TV), and the apparent densities (ρ^{app}) due to dehydration.

It follows that the reduction in bone volume fraction ($\Delta BV/TV$) for each specimen can be calculated from:

$$\frac{\Delta BV}{TV} = \frac{BV/TV_{hyd} - BV/TV_{dry}}{BV/TV_{hyd}} \tag{6}$$

and the increase in the apparent elastic modulus (ΔE^{app}) can be obtained from:

$$\Delta E^{app} = \frac{E_{dry}^{app} - E_{hyd}^{app}}{E_{hyd}^{app}}. \tag{7}$$

Power-law functions are commonly adopted to relate apparent elastic modulus (E) to bone volume fraction (BV/TV) [6,14,15]. If the hydrated (hyd) and dehydrated (dry) data are treated independently, then these relationships can be described using:

$$E_{hyd} = a_{hyd}(BV/TV_{hyd})^{b_{hyd}}, \qquad E_{dry} = a_{dry}(BV/TV_{dry})^{b_{dry}} \tag{8}$$

where the a and b terms are independent constants. A least-squares fitting of Eq. (8) was performed using numerical computer software (MatLab, The Mathworks).

Unfortunately, the use of Eq. (8) results in a ratio of the dehydrated and hydrated apparent moduli (E_{dry}/E_{hyd}) which will vary as a function of BV/TV. Since the ratio of the moduli corresponds to the increase in apparent modulus due to changes of the tissue properties (Fig. 1), it also means the increase in tissue modulus cannot be treated as constant. However, if the data is not treated independently, but is fit to equations of the form:

$$E_{hyd}^{const} = a(BV/TV_{hyd})^{b}, \qquad E_{dry}^{const} = c(BV/TV_{dry})^{b} \tag{9}$$

where the exponent b is constrained to be identical for both equations, then the increase in the tissue modulus (ΔE_{tiss}) is a constant given by:

$$\Delta E_{tiss} = \frac{E_{dry}^{const} - E_{hyd}^{const}}{E_{hyd}^{const}} = \frac{c}{a} - 1. \tag{10}$$

This is similar to the argument made by van Rietbergen et al. [34], who have shown that the predicted apparent moduli of cancellous bone micro-finite element method models scale linearly with the assigned isotropic elastic tissue modulus. Least-squares fitting routines in MatLab were again used to determine the values of the constants a–c.

Assuming that the net change in apparent modulus (ΔE^{app}) results from an increase due to tissue stiffening (ΔE_{tiss}) and a reduction due to reduced bone volume fraction ($\Delta E_{BV/TV}$), as illustrated in Fig. 1, it follows that:

$$\Delta E_{BV/TV} = \Delta E_{tiss} - \Delta E^{app}. \tag{11}$$

Alternatively, $\Delta E_{BV/TV}$ can be estimated from the $\Delta BV/TV$ and Eq. (9) as:

$$\Delta E_{BV/TV} = \frac{E_{hyd}^{adj}(BV/TV_{hyd}) - E_{hyd}^{const}(BV/TV_{dry})}{E_{hyd}^{const}(BV/TV_{hyd})} = 1 - \left(1 - \frac{\Delta BV}{TV}\right)^{b}. \tag{12}$$

3. Results

Of the 51 prepared specimens, three were excluded due to failure and slippage during mechanical testing. These exclusions were made based on the coefficient of determination of a linear regression analysis relating stress and strain; that is, specimens with coefficients of determination below a prescribed cutoff value ($R^2 < 0.993$) were excluded. Eight additional specimens were excluded because one or more properties (apparent density, average increase in apparent modulus, average decrease in bone volume fraction) were more than three standard deviations away from the mean values of the given properties. As a result, 40 specimens were included in the analysis.

The average percentage changes in the macroscopic specimen dimensions of the extracted cores were very small. The average hydrated height (h_{hyd}) of the cores was 6.00 ± 0.09 mm (mean $\pm$ SD) and dehydration resulted in a decrease in height ($\Delta h = -0.004 \pm 0.230\%$). Diameter showed the reverse effect: the average hydration diameter was 8.19 ± 0.02 mm but increased with dehydration ($\Delta D = 0.06 \pm 0.20\%$). Paired t-tests revealed no statistical differences between the heights ($p = 0.910$) as a function of hydration level, while the diameters ($p = 0.055$) showed a trend toward significance.

The average apparent modulus (E^{app}) was 2.37 ± 0.73 GPa (range: 0.97–3.78 GPa) for the hydrated specimens and 2.71 ± 0.92 GPa (range: 1.12–4.76 GPa) for the dehydrated specimens. The increase in apparent modulus due to dehydration was calculated for each specimen using Eq. (7) and the average value was $14.3 \pm 14.3\%$ (mean equal to the standard deviation, range: −18.7–55.8%). The average bone volume fraction (BV/TV) was 0.278 ± 0.061 (range: 0.159–0.425) for the hydrated specimens and 0.258 ± 0.057 (range: 0.150–0.393) for the dehydrated specimens. The hydrated and dehydrated apparent moduli were $\rho_{hyd}^{app} = 0.53 \pm 0.11\,\mathrm{g/cm^3}$ and $\rho_{dry}^{app} = 0.51 \pm 0.11\,\mathrm{g/cm^3}$, respectively. Paired t-tests reveal that E^{app} ($p < 0.0001$), BV/TV ($p < 0.0001$) and ρ^{app} ($p < 0.0001$) were all significantly affected by dehydration. The shrinkage, or change in bone volume fraction ($\Delta BV/TV$), due

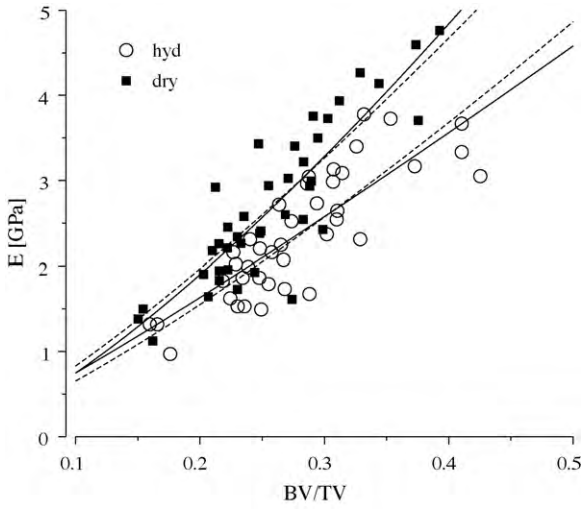

Fig. 2. Apparent elastic modulus (E), for hydrated (hyd) and dehydrated (dry) conditions, plotted against bone volume fraction (BV/TV). The solid lines are those obtained using Eq. (8) while the dashed lines are the curves obtained using Eq. (9), which are constrained to have a constant exponent. The equations and R^2 values for these curves are given in Table 2.

to dehydration was calculated for each specimen and the average value was $7.2 \pm 1.4\%$ (range: 4.8–13.0%).

The hydrated and dehydrated moduli are plotted as a function of bone volume fraction in Fig. 2. The equations and R^2 value for the best fit and constant–exponent curves are given in Table 2. Since both the equations of the form in Eq. (8) (solid lines) and Eq. (9) (dashed lines) give similar results, this supports the assumption of equal-exponents used to derive Eq. (10). Using Eqs. (10) and (11) an average increase in tissue modulus (ΔE_{tiss}) of 26.9% and an average decrease in apparent modulus of 12.6% attributable to shrinkage is estimated. However, the decrease due to shrinkage is found to be 8.9% when Eq. (12) is employed.

4. Discussion

Water is an important contributor to the structure and mechanical behaviour of bone and other calcified tissues. Bone undergoes both structural and tissue-level changes during dehydration which combine to alter the apparent mechanical properties. This study has isolated these contributions in bovine cancellous bone using power-law regressions that relate apparent modulus (E) to bone volume fraction (BV/TV) in both the hydrated and dehydrated states.

Based on Eq. (10), the increase in tissue modulus due to dehydration was estimated to be 26.9%, which is similar to the the 28% increase measured using nanoindentation in trabeculae from the bovine femoral head [2]. Interestingly, both of these results are much higher than the increases reported by Rho and Pharr [27] for interstitial lamellae (9.7%) and osteons (15.4%) in the bovine femur, whereas even larger increases (101.9%) due to dehydration were reported by Akhtar et al. [1] in antler trabeculae from fallow deer. Since the average mineralization decreases, in order, from interstitial lamellae to osteons to trabeculae to antler, the current results suggest that dehydration has a greater effect in less mineralized tissues [10,1,28]. An inverse relationship between water content and mineralization has long been recognized [30,12]. Therefore, it is expected that the mechanical properties of less mineralized tissues (e.g. trabeculae) will undergo greater changes during dehydration than more mineralized tissues (e.g. interstitial lamellae) because a greater fraction of water is removed from the former.

Table 2
Equations relating apparent modulus (E) to bone volume fraction (BV/TV), in hydrated (hyd) and dehydrated (dry) conditions, using either the curves of Eq. (8) or the curves of Eq. (9) which were constrained to have a constant exponent.

Equation	R^2
$E_{hyd} = 10.0(BV/TV_{hyd})^{1.13}$	0.70
$E_{dry} = 16.6(BV/TV_{dry})^{1.35}$	0.77
$E_{hyd}^{const} = 11.6(BV/TV_{hyd})^{1.25}$	0.69
$E_{dry}^{const} = 14.7(BV/TV_{dry})^{1.25}$	0.76

An average decrease in bone volume fraction of 7.2% was measured after dehydration of the samples. Lievers et al. [20] recently measured a reduction in bone volume fraction of approximately 16% due to dehydration for a range of specimen diameters. When equivalent diameters (8.3 mm) are compared, values of 7% versus 12% are obtained. Part of the discrepancy in these numbers may be attributed to differences in anatomic site. Lievers et al. [20] examined specimens from the bovine femoral condyle, which has a more isotropic trabecular architecture than the preferential cranial–caudal orientation of trabeculae in the bovine lumbar vertebrae examined herein. Architecture will likely affect the amount of macroscopic shrinkage.

The reduction in apparent modulus due to the decrease in bone volume fraction alone was calculated to be 12.6% based on Eq. (11); however, when calculated from the measured average reduction in bone volume fraction ($\Delta BV/TV$) and Eq. (12), the decrease is only 8.9%. The discrepancy between these two values underscores the fact that they are estimates of the contributions. Nevertheless, the relative agreement between the two ($\Delta E_{BV/TV}$) values suggests that they are useful indicators of the magnitude of the changes that occur.

The power-law exponents in the equations relating modulus (E) and bone volume fraction (BV/TV) were somewhat lower than expected. Typically, values can be expected in the range 1.5–2.5 [29,24] for a variety of species and anatomic site combinations, whereas the current work obtained values of 1.13–1.35. A previous study by the authors [21], also involving cancellous bone from bovine vertebrae, did obtain similar results in 8.3 mm diameter specimens ($E = 7.91 BV/TV^{0.88}$ GPa). Although the reasons for these lower exponent values are unclear, it might be attributable to the strong preferential orientation of the trabeculae in the bovine spine.

One conceivable source of error is damage to the specimens due to repeated mechanical loading and cycling between hydrated and dehydrated states. Unfortunately, these conditions were unavoidable in order to gather the information needed to estimate the magnitude of the structural effects and changes in apparent elastic modulus. The large range and standard deviation associated with the increase in apparent modulus due to dehydration ($\Delta E^{app} = 14.3 \pm 14.3\%$) may at first appear to suggest problems with the testing protocol. It must be reiterated, however, that ΔE^{app} is the net effect of both geometric and material level changes. Since these changes themselves have variability, the variability of their interaction is even greater, as would be expected from error propagation analysis. A final potential concern is the fact that shrinking trabeculae could lead to a loosening of the epoxy–bone interface during dehydration, although no evidence of this behaviour was noted. The removal of marrow prior to, and the time required during, embedding probably resulted in some degree of trabecular dehydration which reduced the danger of loosening.

Although the magnitude of the tissue-level changes is roughly 2–3 times that of the structural changes, the current study suggests that both effects must be accounted for when modeling the effects of cancellous bone dehydration. For example, Akhtar et al. [2] used the average hydrated and dehydrated nanoindentation moduli as the tissue modulus inputs to finite element method (FEM) models. Because the same FEM meshes were employed for both the

hydrated and dehydrated simulations, the models did not account for the structural changes that also occurred ($\Delta BV/TV$). As a result, the ratio of the hydrated/dehydrated apparent moduli was equivalent to the ratio of hydrated/dehydrated nanoindentation-derived tissue moduli (as would be expected from [34]) due to the failure to account for changes in architecture.

The current study has treated the structural changes as isotropic; however, dehydration-induced shrinkage in bone has been shown to be anisotropic at the macroscopic [13] and at the microstructural level [33]. This anisotropy will result in architectural changes that cannot be captured using the methodology described herein. Micro-CT imaging, performed under both hydrated and dehydrated conditions, could be used in future studies to quantify both the change in BV/TV and the change in architecture. Furthermore, three-dimensional imaging could be combined with local mineralization measures to examine the effect of mineral heterogeneity on these dehydration-induced architectural changes. It might also be possible, using hydrated testing performed at both room temperature and at 37 °C, to induce a change in BV/TV without altering the hydration level; however, it remains unclear whether the change in modulus or BV/TV would be significant over that temperature range [8,4,23]. Nevertheless, these approaches could provide further insight into the cooperative role of water and mineral on the structural and mechanical behaviour of calcified tissues.

In summary, the current work has shown that the measured increase in the apparent modulus of cancellous bone following dehydration (14.3%) results from a combination of both structural and material changes. The increase in tissue modulus was estimated to increase the apparent modulus by 26.9%, while the reduction in bone volume fraction caused a decrease in apparent modulus of approximately 12.6%. Therefore, it is imperative that both structural and material changes are considered when studying the role of water in determining the mechanical properties of bone.

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Conflict of interest

The authors have no conflict of interest.

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